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$GABA_B$-Mediated Presynaptic Inhibition of Excitatory Transmission and Synaptic Vesicle Dynamics in Cultured Hippocampal Neurons

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Summary

Local recycling of synaptic vesicle membrane at nerve terminals is necessary to maintain a readily releasable pool of transmitter. To what extent are the dynamics of vesicle recycling subject to modulation? We examined the influence of presynaptic $GABA_B$ receptors on vesicle dynamics at single synapses using optical imaging of FM1–43 in cultured rat hippocampal neurons. The kinetics of FM1–43 destaining indicate that synapses from a single neuron have a unimodal distribution of release probabilities, and $GABA_B$-mediated inhibition occurs uniformly at all sites. Electrical and optical recordings from single cells show that the inhibition of excitatory transmission is entirely accounted for by a rapidly reversible reduction of exocytosis. In contrast, $GABA_B$ receptors do not alter the rate or extent of endocytosis.

Introduction

Although much is known about properties of postsynaptic neurons in the mammalian brain, processes directly regulating transmitter release at presynaptic terminals and their modulation remain less understood. In the mammalian central nervous system, methods based on quantal analysis of postsynaptic responses have revealed factors regulating transmitter release (Redman, 1990). However, this approach does not distinguish between mechanisms such as the modulation of presynaptic ion channels or direct modulation of the exocytotic machinery. Neurotransmitter exocytosis occurs at discrete active zones containing readily releasable pools of synaptic vesicles. Transmitter release requires fusion of synaptic vesicles with the plasma membrane of the nerve terminal, and this membrane is retrieved and recycled for subsequent rounds of transmitter release (Scheller, 1995). These crucial steps in transmitter release can now be studied more directly using optical imaging of a fluorescent membrane probe, FM1–43, developed by Betz and colleagues at the neuromuscular junction (Betz and Bewick, 1992; 1993; Betz et al., 1992) and recently applied to cultured hippocampal neurons (Ryan, et al., 1993; 1996a; Ryan and Smith, 1995).

Many G-protein-coupled neurotransmitter receptors, including those activated by GABA, acetylcholine, glutamate, and adenosine, modulate synaptic transmission by direct effects (usually inhibitory) on nerve terminals (Nicoll et al., 1990; Thompson et al., 1993). Presynaptic voltage-gated calcium channels are thought to represent an important target in the modulation of transmitter release (Wu and Saggau, 1995; Dittman and Regehr, 1996), and virtually all neuromodulators that block transmitter release have been shown to inhibit somatic calcium channels in a variety of central neurons (Nicoll et al., 1990; Anwyl, 1991). Nevertheless, patch-clamp studies of synaptic transmission have revealed that activation of presynaptic receptors also inhibits calcium-influx-independent spontaneous transmitter release (Scholz and Miller, 1992; Scanziani et al., 1992; Umemiya and Berger, 1995; Scanziani et al., 1995; Dittman and Regehr, 1996; Capogna et al., 1996). Such results raised the hypothesis that presynaptic receptors exert an important effect on transmitter release at a step downstream of calcium influx. Thus, the precise presynaptic mechanisms of action of most neuromodulators remain unclear.

To explore mechanisms of action of neuromodulators further, we have examined to what extent presynaptic receptors regulate synaptic vesicle recycling. Maintenance of a readily releasable pool of vesicles is an important process governing synaptic strength (Rosenmund and Stevens, 1996) and would be severely affected by down-regulation of the rates of endocytosis or vesicle "repriming." In this study, we have examined the actions of the $GABA_B$ receptor agonist, baclofen, on exo- and endocytosis in hippocampal neurons grown on single-cell microislands or in mass culture. We find that $GABA_B$ receptors inhibit exocytosis uniformly at all synapses but do not markedly inhibit endocytosis. We also show, using simultaneous voltage-clamp recording of autaptic transmission and FM1–43 imaging, that the inhibition of excitatory transmission can be entirely accounted for by the inhibition of vesicle exocytosis. Some experiments in hippocampal neurons have suggested that the probability of transmitter release is nonuniform at different release sites (Hessler et al., 1993; Rosenmund et al., 1993). These studies relied on a postsynaptic receptor blocker to determine presynaptic release probability indirectly. Using the rate of FM1–43 destaining at individual synapses as a measure of synaptic vesicle release, we have determined directly the range of release probabilities across multiple release sites arising from the same axon.

Results

Synaptic vesicle membrane can be selectively labeled with the fluorescent dye FM1–43 in an activity-dependent manner. We examined synaptic vesicle dynamics in neurons cultured from regions CA1-CA3 of the rat hippocampus. Field stimulation of hippocampal cultures in the presence of FM1–43 followed by washing of the dye labels discrete puncta corresponding to recently retrieved synaptic vesicle membrane at release sites (Figure 1A) (Reuter, 1995; Reuter and Porzig, 1995; Ryan and Smith, 1995; Ryan et al., 1996a; 1996b). Virtually all of this trapped fluorescence is released following a second round of stimulation (20 Hz for 90 s) in control solution (Figure 1B). Consistent with a release mechanism that depends on action potentials and calcium channels, stimulus-evoked destaining was prevented by

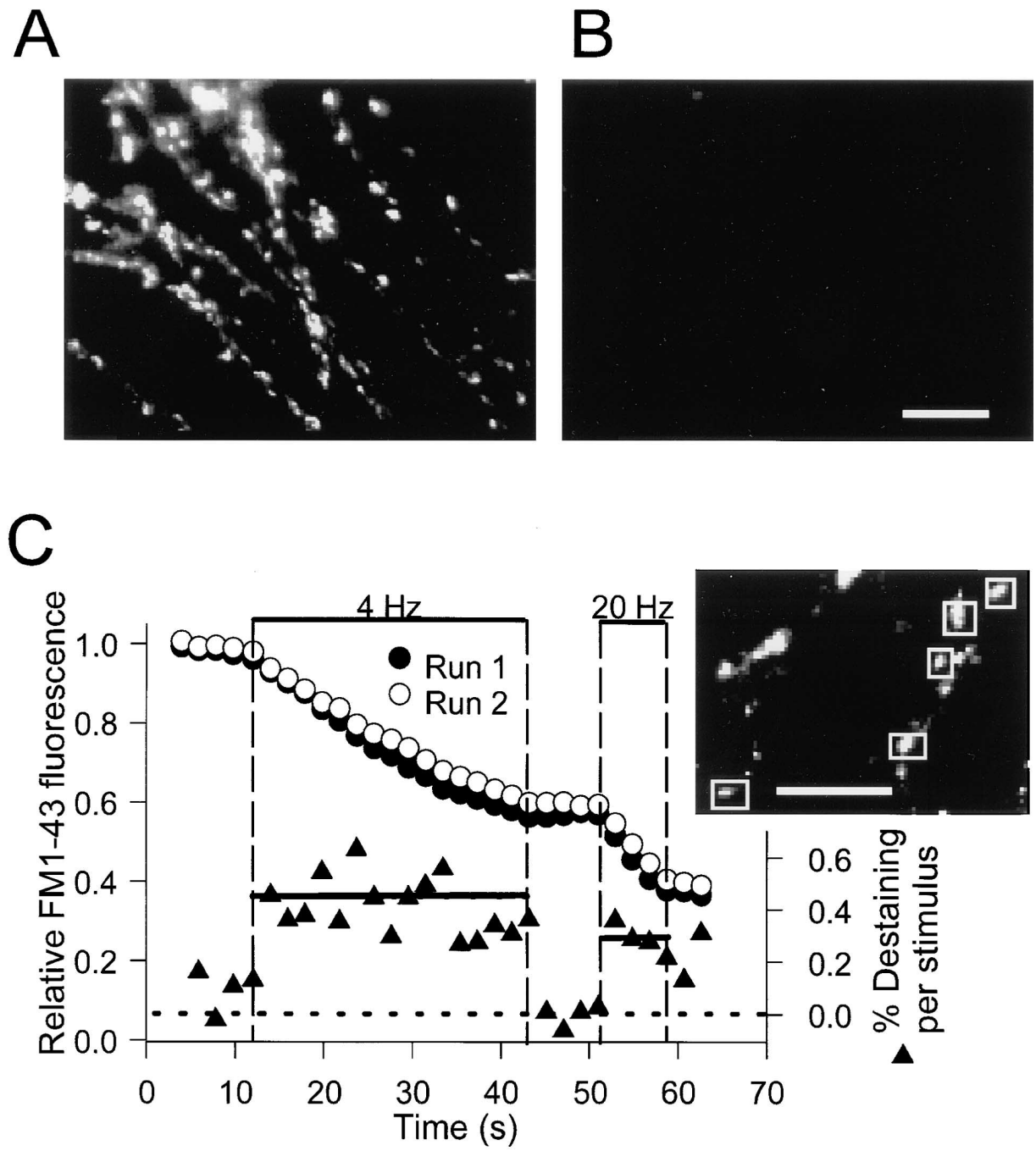

Figure 1. FM1–43 Destaining of Presynaptic Boutons Is Repeatable

(A) Fluorescence image of neurites from hippocampal neurons grown in mass culture and stained with FM1–43 by 20 s of field stimulation at 20 Hz. Each bright spot presumably represents a cluster of recycled vesicles at an active zone.

(B) Same field of view as shown in (A) immediately following 90 s of stimulation at 20 Hz in the absence of dye. Digital images in (A) and (B) were processed identically. Scale bar = 10 μm.

(C) Average time course of fluorescence for 10 synapses in one field (closed circles) and the average time course for the same 10 synapses after reloading with FM1–43 (open circles). Destaining rate constant per stimulus (triangles) represents the average values of these two runs. Horizontal lines represent the average rate constants within each stimulus period. In the absence of stimulation, rate constants represent destaining per 250 ms. Inset shows five of the synapses analyzed in this experiment outlined by boxes. For clarity, boxed regions are larger than the areas used for measurement of fluorescent intensity. Scale bar = 10 μm.

adding tetrodotoxin (1 μM) or cadmium chloride (200 μM) to the perfusing solution (data not shown). The time course of exocytosis can be studied by measuring the destaining during stimulation in dye-free solution (Figure 1C), and when the same boutons are loaded and destained twice, the rate of exocytosis is identical in the

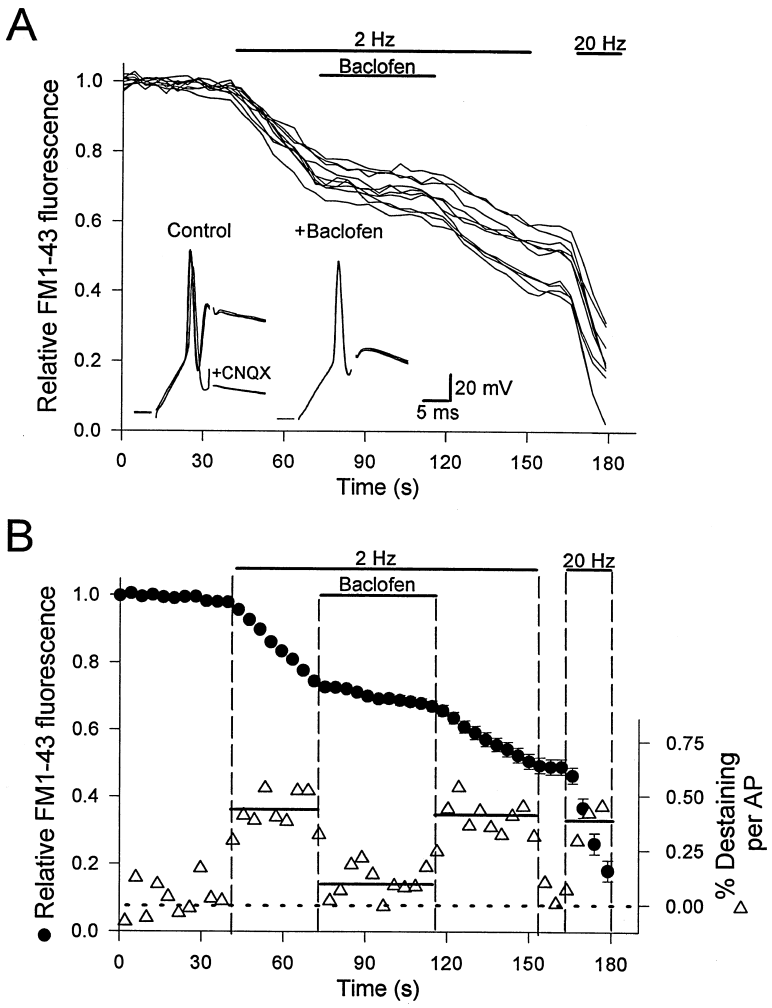

Figure 2. FM1–43 Destaining and Synaptic Transmission Recorded from One Cell

(A) Relative FM1–43 fluorescence of 10 synapses from the same cell. Baclofen (50 μM) markedly slowed the rates of destaining at every synapse. Action potentials were elicited by a 10 ms, 0.1 nA current pulse; V_m = −80 mV. Insets show sets of three consecutive potential recordings under several conditions. Series resistance was digitally compensated, and capacitative artifacts have been blanked for clarity. Under control conditions, the action potential was followed by a large autaptic depolarizing potential (Control). Prior to the destaining measurements, a brief application of CNQX (20 μM) abolished the autaptic response (+CNQX, superimposed on the control records). To the right, baclofen reduced the amplitude of the EPSP but did not inhibit the generation of action potentials (+Baclofen).

(B) Average relative FM1–43 fluorescence (closed circles) and destaining rate per action potential (open triangles) of the same 10 synapses. Horizontal lines through the data show the average destaining rate constants during periods delineated by the vertical dashed lines. In the absence of stimulatiom, rate constants represent destaining per 500 ms.

two destaining runs. These results confirm that destaining rates are reproducible over multiple periods of stimulation (Reuter, 1995). Destaining at stimulus frequencies of 2–4 Hz averaged ~0.5% of the recycling vesicle pool per stimulus. As expected, increasing the stimulus frequency to 20 Hz caused an increase in the relative rate of destaining; however, the rate constant per stimulus was somewhat reduced (Figure 1C).

Activation of presynaptic $GABA_B$ receptors potently inhibits synaptic transmission at a variety of central synapses (Mott and Lewis, 1994). Therefore, we examined the action of the $GABA_B$ agonist baclofen on synaptic transmission and on FM1–43 destaining at excitatory synapses from single cells grown in microisland culture. Under these conditions, an isolated neuron makes synapses with itself ("autapses"); thus, autaptic currents/potentials are monosynaptic, and all inputs are derived from the same cell. The results from one cell recorded in current clamp are shown in Figure 2. The cell was held at a resting potential of −80 mV, and a depolarizing current step was injected to elicit a single action potential (Figure 2A inset). The action potential was followed by a large excitatory postsynaptic potential (EPSP) that was blocked by the non-N-methyl-D-aspartate (non-NMDA) antagonist CNQX, confirming that the EPSP was mediated by glutamate receptors. The cell was loaded with FM1–43 by a round of stimulation, washed free of the dye, and then stimulated again to study the kinetics of destaining. Baclofen (50 μM) depressed the amplitude of the autaptic EPSP and markedly slowed the rate of destaining at each of the synapses studied in this cell (Figure 2A). Synapses from this cell showed striking similarity in the relative rate of destaining despite the fact that their initial raw fluorescence values (not shown) varied over a 2-fold range. In this experiment, the destaining rate constant per action potential averaged 0.43% under control conditions at 2 Hz stimulation and fell to 0.10% in the presence of baclofen (Figure 2B). Following washout of baclofen, the rate recovered to 0.40% and was somewhat less during a subsequent period of 20 Hz stimulation (0.39%). These results indicate that baclofen dramatically decreases the rate of exocytosis as measured by FM1–43 dye loss.

We next compared the presynaptic inhibition of vesicle release and excitatory transmission in the same neurons. In these experiments, cells were voltage clamped to monitor autaptic excitatory postsynaptic currents (EPSCs) (Figure 3, top). Baclofen caused a rapid and reversible inhibition of EPSC amplitude (control amplitude 3.0 ± 1.7 nA), which averaged 73% ± 4% (n = 6).

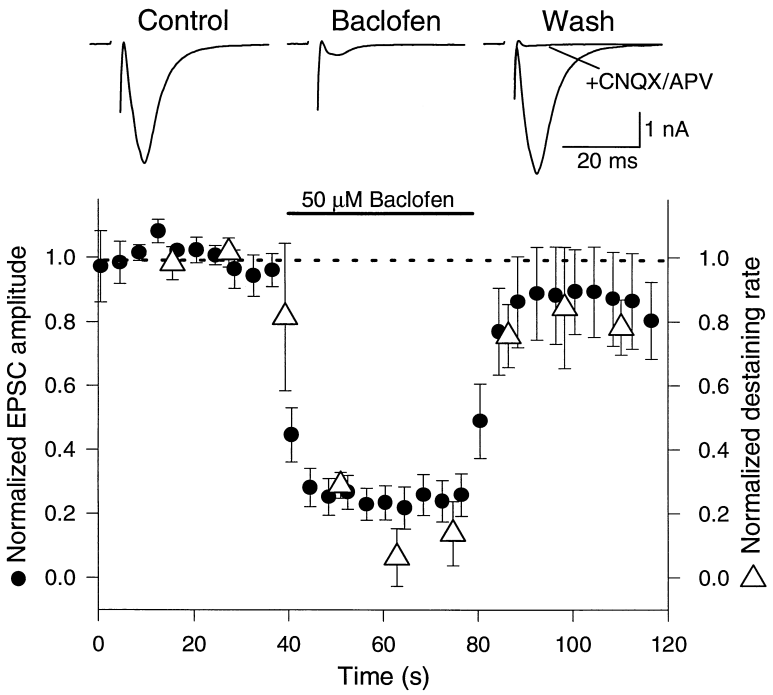

Figure 3. Time Course and Extent of Inhibition of Autaptic EPSCs and FM1–43 Destaining Are the Same

Top: An average of five consecutive autaptic EPSCs under control conditions, in the presence of baclofen (50 μM) and following washout. The EPSC is abolished by the subsequent application of CNQX (20 μM) and D-APV (25 μM). The graph shows relative inhibition of EPSC amplitude in response to baclofen (50 μM, closed circles; n = 6). Each point is the average of eight consecutive EPSCs evoked at 2 Hz normalized to the average of all points before application of baclofen. Plotted on the same graph is the relative destaining rate constant from the same cells (open circles; n = 7, includes 1 cell recorded in current clamp). Each point is the mean rate from three successive images and normalized to the destaining rate prior to application of baclofen.

There was only a small and variable action of baclofen on membrane holding current, averaging +14 ± 18 pA (n = 8). In seven cells (six in voltage clamp, one in current clamp), the destaining rate constant averaged 0.65% ± 0.12% (range 0.4–1.21) per stimulus under control conditions. This was reduced to 0.12% ± 0.01% in the presence of baclofen and recovered to 0.56% ± 0.08% upon washout of the agonist. Both the rapid time course and the extent of inhibition of FM1–43 destaining were identical to that measured by the EPSC (Figure 3). These results indicate that FM1–43 destaining faithfully tracks the strength of synaptic transmission in single axons.

Several studies have indirectly examined the probability of transmitter release (P_r) by monitoring the progressive blockade of NMDA receptor–mediated EPSCs by the irreversible open channel blocker MK-801 (Hessler et al., 1993; Rosenmund et al., 1993). Those results suggested that P_r was nonuniform across different synapses and in fact fell into two classes: a small subset of high P_r synapses and a much larger population of low P_r synapses. We studied the initial rates of FM1–43 destaining at individual release sites as a measure of the rate of release of synaptic vesicles to determine more directly the range of P_r from sites arising from the same neuron. Initial destaining rates were determined by linear fits to the dye loss measured during the first 20 s of stimulation at 2 Hz. Destaining rates for boutons from autaptic recordings (~10/cell) were normalized with respect to the mean destaining rate from the same cells. The initial destaining rates were well fitted by a single Guassian distribution (coefficient of variation = 33%; n = 55 synapses), indicating that under our recording conditions (~14 days in vitro), there was no evidence for two distinct populations of synapses (Figure 4), and the distribution of P_r values is fairly broad.

We also studied the action of baclofen in mass cultures. As in the autaptic recordings, baclofen caused a marked inhibition of destaining evoked by 4 Hz field stimulation (Figure 5A; n = 4 cultures). Similar to our single-cell measurements, under control conditions, the average destaining rate constant during 4 Hz field stimulation was 0.61% ± 0.03%/stimulus, and in the presence of baclofen, the rate constant was reduced to 0.11% ± 0.02%/stimulus. Curiously, however, the inhibition was frequency dependent. Subsequent higher frequency stimulation (20 Hz) in the same baclofen-treated cells evoked destaining at rates approaching those observed under control conditions (20 Hz control: 0.37% ± 0.02%/

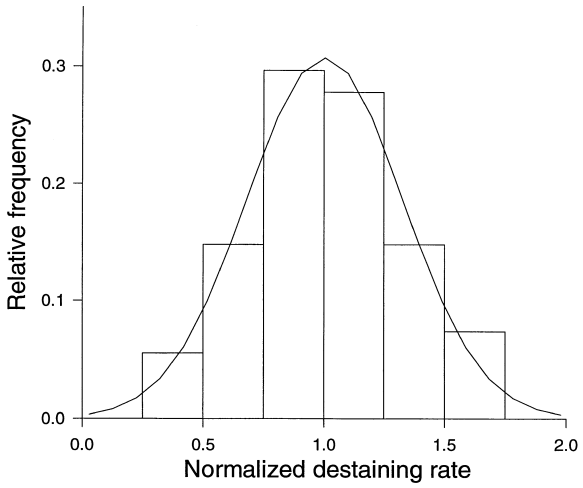

Figure 4. Destaining Rate Constants of Many Individual Synapses Suggest a Single Population of Release Probabilities

Histogram of initial destaining rates (2 Hz stimulation) from six cells normalized to the average rates from each cell (n = 55 synapses). The line is a single Guassian fitted to the data with a coefficient of variation of 33%.

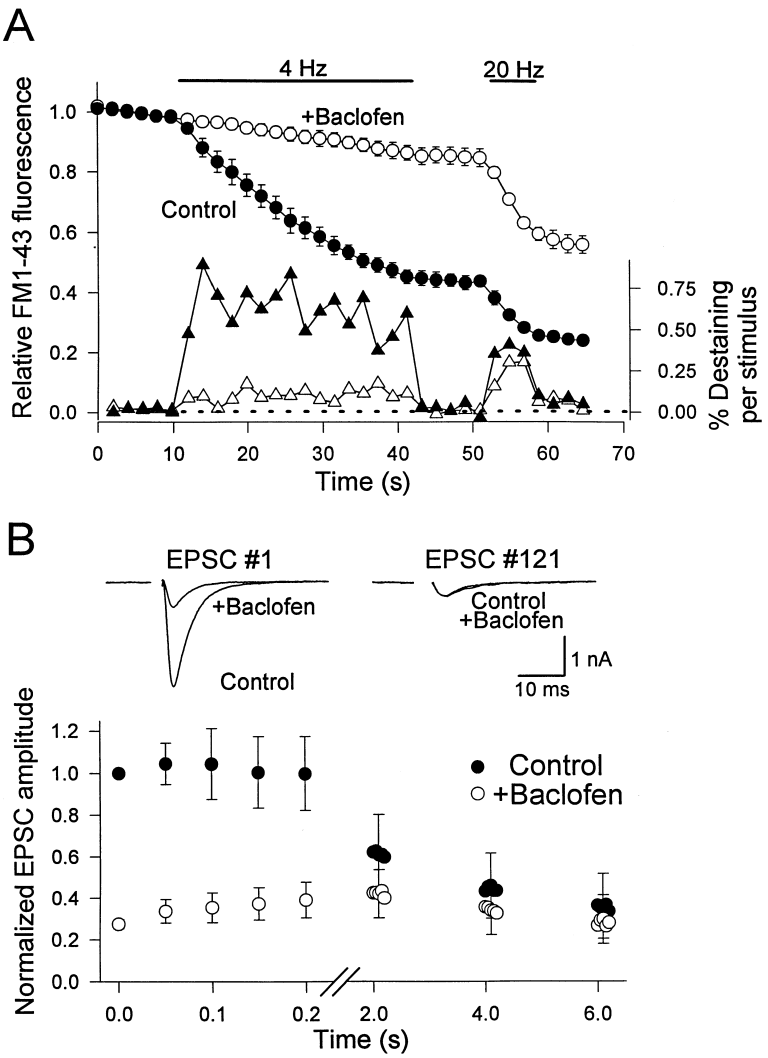

Figure 5. Frequency Dependence of Baclofen Inhibition of FM1–43 Destaining and Synaptic Transmission

(A) Baclofen strongly inhibits field-stimulation-evoked destaining at 4 Hz relative to control but is much less effective at 20 Hz (n = 4 cultures). Destaining under control conditions (closed circles) and the same synapses in the presence of baclofen (50 μM, open circles). Triangles show destaining rate per stimulus. In the absence of stimulation, rate constants represent destaining per 50 ms.

(B) Baclofen is less effective at inhibiting synaptic transmission evoked at 20 Hz. Autaptic currents were evoked at 20 Hz for 6.5 s every 35 s. Each point represents the average of 3–5 traces under control conditions (open circles) and in the presence of baclofen (closed circles, 50 μM; n = 5 cells). Results are plotted for the first five EPSCs in the train and for five EPSCs evoked at 2, 4, and 6 s into the stimulus. All points are normalized with respect to the amplitude of the first EPSC under control conditions. Upper traces show representative responses from one cell.

stimulus; 20 Hz baclofen: 0.25% ± 0.05 %) (Figure 5A). Similarly, in other experiments, destaining evoked by 20 Hz stimulation alone was only modestly inhibited by baclofen.

To further address the frequency-dependent action of baclofen, we examined its effects on autaptic EPSCs evoked at a frequency of 20 Hz. Without baclofen, the EPSC amplitude gradually declined during a 6 s train of 20 Hz stimulation (Figure 5B) and at the end of the train averaged 36% ± 16% (n = 5) relative to the first EPSC of the train. Such depression of transmitter release is often ascribed to depletion of the presynaptic vesicle pool. In the presence of baclofen, the amplitude of the first EPSC of a 20 Hz train was 27% ± 2% of the first control response, and 6 s later, it still averaged 30% ± 12% (Figure 5B). As expected, baclofen reduced the first EPSC strongly (by 73%); however, unlike the control condition, there was no additional depression of the EPSCs during the 6 s of high frequency stimulation. Therefore, the EPSC amplitudes with and without baclofen differed by only 17% at the end of the train. This effect is in the same direction as was found with FM1–43, where baclofen caused an average reduction of 82% of the destaining rate during 4 Hz stimulation and 32% during 20 Hz stimulation (Figure 5A). Taken at face value, however, our data suggest that even without baclofen, 20 Hz stimulation depresses the EPSCs more than it depresses FM1–43 destaining. An interesting speculative interpretation would be that some of the membrane cycling measured by FM1–43 at high frequencies is less effective at delivering transmitter to receptors (due to incomplete filling of vesicles), or that some receptors are desensitizing.

In addition to inhibiting nerve-evoked transmission, baclofen causes a marked reduction in the frequency of TTX- and calcium-influx-independent miniature EPSCs (Scholz and Miller, 1992; Scanziani et al., 1992; Capogna et al., 1996). This has raised the suggestion that G-protein-coupled presynaptic receptors inhibit synaptic vesicle dynamics at steps downstream of calcium influx. Given this hypothesis, we next made FM1–43 pulse-chase measurements (Ryan and Smith, 1995; Ryan et al., 1996a) of the endocytic phase of the synaptic vesicle cycle to determine whether $GABA_B$ receptors modulate events underlying endocytosis in mass cultures of hippocampal neurons.

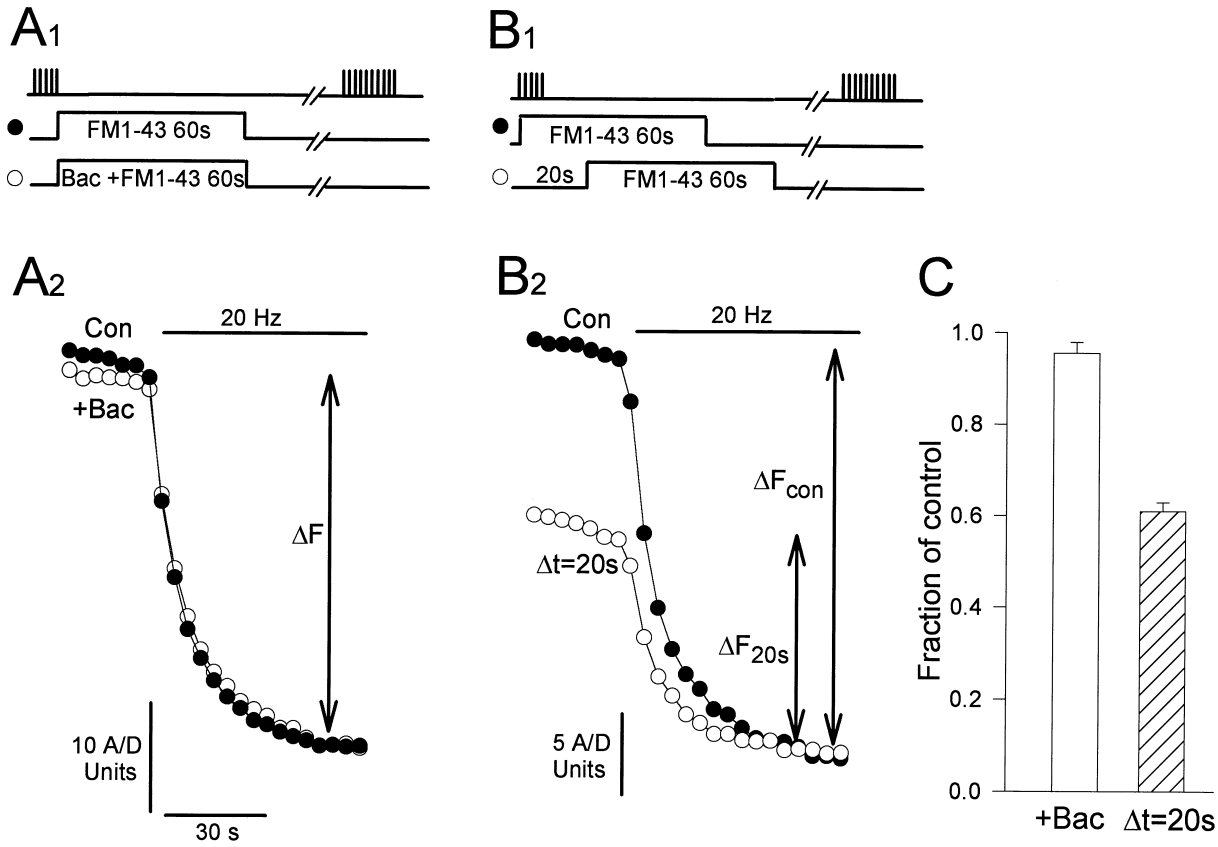

Figure 6. Baclofen Does Not Inhibit Endocytosis

(A1) Protocol. In one set of experiments, exocytosis is initiated by a brief period of stimulation (20 Hz for 5 s) immediately followed by the application of FM1–43 for 60 s. Following 5 min of washing, the size of the labeled pool of synaptic vesicles is determined by measuring the fluorescence (ΔF) released by a second period of stimulation (20 Hz for 90 s). The size of the pool labeled under these control conditions is then compared to the size of the pool labeled by the same protocol except baclofen (50 μM) is coapplied with FM1–43.

(A2) Representative experiment examining the action of baclofen on endocytosis. Membrane retrieval (measured as ΔF) in the presence of baclofen (open circles) is not significantly different from control (closed circles). In all endocytosis experiments, correction for effects of gradual rundown was made by repeating the control protocol after the test run. Control ΔF measurements represent the average of these two bracketing control runs.

(B1) In another set of experiments, exocytosis is triggered (20 Hz for 5 s) at the start of a 60 s application of FM1–43. The size of the pool labeled under these control conditions is then compared to that when the 60 s application of FM1–43 is delayed by 20 s from the onset of stimulation.

(B2) Representative experiment shows that a change in endocytosis could be detected with the protocol shown in (B1). The size of the recycling pool labeled under control conditions (filled circles) is significantly greater than the pool labeled with a 20 s delay from the onset of stimulation.

(C) Average labeling relative to control for the effect of baclofen (protocol [A1]; n = 6 cultures) and the delayed application of FM1–43 (protocol [B1]; n = 4 cultures).

To study the action of baclofen on endocytosis, we used dye-loading protocols designed to measure membrane retrieval for 60 s following a brief period of exocytosis (20 Hz for 5 s) (Figures 6A1 and 6B1). Previous results indicate that the majority of recycling vesicles (~80%) are retrieved within 60 s of such a brief stimulus (Ryan et al., 1996a). The pool of vesicles undergoing endocytosis was labeled by applying FM1–43 for 60 s immediately after the end of the 5 s stimulation. Following washout of the dye for 5 min, a second round of stimulation was delivered to measure the size of the pool of vesicles that had undergone endocytosis during the 60 s poststimulus period. The size of this pool was taken as the absolute amount of fluorescence released (ΔF) during 90 s of 20 Hz stimulation (Figure 6A2). The same synapses were then loaded with FM1–43 in the presence of baclofen for 60 s, and ΔF was compared to that measured under control conditions in the same synapses. Baclofen did not significantly depress the extent of endocytosis in any of the cultures examined (n = 6; Figure 6A2).

Because the previous result was negative, we wanted to validate our method by showing that we can detect changes in ΔF when they occur. We chose to reduce ΔF by delaying the application of the FM1–43. In the control run for these experiments, dye was applied for 60 s beginning with the onset of the brief stimulus to trigger exocytosis. The ΔF from this loading protocol was compared to that from the same synapses when the FM1–43 application was delayed for 20 s from the

start of stimulation (Figure 5B1). Any vesicles undergoing endocytosis during this 20 s interval will escape labeling and would therefore not contribute to the recycling pool measured during the subsequent staining–destaining protocol. As expected, following a 20 s delay, ΔF was significantly less than control (Figure 6B2). On average, it was 39% $\pm$ 2% (n = 4 cultures) smaller (Figure 6C). These results indicate that we can detect changes in ΔF and support our findings that baclofen did not inhibit endocytosis over the 60 s period when the majority of vesicle membrane is retrieved.

While baclofen did not inhibit the extent of endocytosis, it might still slow the rate of endocytosis. To test for this possibility, we studied the action of baclofen at an early point in the time course of membrane recycling. In these experiments, a 20 s delay was again imposed between the beginning of the brief loading stimulus (20 Hz for 5 s) and the start of a 60 s application of FM1–43. The ΔF from this loading protocol without baclofen was compared to that from the same synapses when baclofen was included during the 15 s immediately following the loading stimulus. In this protocol, if baclofen reduced the rate of endocytosis, fewer vesicles would be retrieved in the 15 s interval when dye was absent, and therefore more would be available to contribute to the labeled pool. Under these conditions, ΔF was not signficantly affected by baclofen (baclofen/control ΔF = 112% $\pm$ 12%; n = 4). Taken together, our results show that baclofen does not markedly alter either the rate or extent of endocytosis.

Discussion

We used the membrane probe FM1–43 to examine the impact of presynaptic inhibition on synaptic vesicle turnover in cultured hippocampal neurons. We studied vesicle dynamics in mass cultures of hippocampal neurons and in synapses arising from the same axons in single excitatory cells. All synapses had a remarkably uniform rate constant of release, and the $GABA_B$ agonist baclofen uniformly inhibited vesicle release at synapses labeled by FM1–43 without depressing endocytosis.

The styryl dye FM1–43 labels recycling synaptic vesicle membranes (Betz et al., 1992; Betz and Bewick, 1993; Henkel and Betz, 1996). It has been used to probe the kinetics of vesicle dynamics in cultured hippocampal neurons (Ryan et al., 1993; 1996a; Ryan and Smith, 1995), including the block of vesicle release at individual synapses by calcium channel antagonists (Reuter, 1995), the influence of the Na^+-Ca^{2+} exchanger (Reuter and Porzig, 1995), and the potentiation of vesicle turnover following tetanic stimulation (Ryan et al., 1996b). All of the studies in hippocampal neurons relied on field stimulation to evoke synaptic vesicle turnover; however, the relationship between vesicle release and classical measures of synaptic transmission (i.e., electrical recordings of nerve-evoked EPSCs) has not been well established. Furthermore, the action of presynaptic receptors on synaptic vesicle dynamics has not been described.

We chose to use baclofen for several reasons. First, presynaptic $GABA_B$ receptors potently inhibit excitatory and inhibitory synaptic transmission in cultured hippocampal cells (Harrison, 1990; Scholz and Miller, 1991; Wilcox and Dichter, 1994; Pfrieger et al., 1994). Second, baclofen inhibits the frequency of calcium-influx-independent miniature EPSCs (Scholz and Miller, 1992; Scanziani et al., 1992), raising the possibility that presynaptic $GABA_B$ receptors interact directly with components of the synaptic vesicle recycling pathway.

We found that baclofen strongly inhibits autaptic EPSCs from cells grown in microisland cultures. Simultaneous optical recording of FM1–43 destaining provided a novel measure of the exocytosis of synaptic vesicles from the same population of synaptic terminals. At the neuromuscular junction, the initial rate of FM1–43 destaining is proportional to synaptic transmission measured by intracellular recordings of end plate potentials (Betz and Bewick, 1993). In agreement with these findings, we have found at a central synapse that the inhibition by baclofen of FM1–43 destaining rate is proportional to the inhibition of EPSCs. These results provide further evidence that nerve-evoked destaining of terminals loaded with FM1–43 faithfully tracks the kinetics of neurotransmitter release and show that the presynaptic inhibition of synaptic vesicle exocytosis may entirely account for the inhibition of evoked transmission.

We found no evidence in either mass cultures or autaptic recordings for discrete populations of FM1-43-labeled synapses that were not inhibited by baclofen during stimulation at 2–4 Hz. In contrast, the N-type calcium channel blocker ω-conotoxin GVIA has been reported to inhibit FM1–43 destaining (evoked by 20 Hz field stimulation) preferentially at a subset of synapses in cultured hippocampal neurons (Reuter, 1995). Despite the potential heterogeneous distribution of presynaptic calcium channel subtypes, we find that $GABA_B$ receptor-mediated inhibition is quite homogeneous.

We have found with 2 Hz stimulation that the rates of destaining from synapses arising from the same axon could be well described by a unimodal distribution of P_r. This seemed surprising, given conclusions drawn using MK-801 blockade of NMDA receptor-mediated EPSCs in similar cultures (Rosenmund et al., 1993). Such studies showing that the progressive block of NMDA EPSCs was well fitted with two exponentials had suggested that there are two populations of sites: those with a high P_r (0.5) and those with a much lower P_r (0.1) that account for $\sim$80% of all synapses; a distinct population of synapses with high P_r was sensitive to inhibition by baclofen. Given our typical dye-loading stimulus of $\sim$250 action potentials, it would seem unlikely that synapses even with a very low P_r would go undetected. Recently, it has been reported (Tovar et al., 1995, Soc. Neurosci. Abstract) that although the progressive block of NMDA EPSCs is fitted with two exponentials at early stages of development ($\sim$7 days in vitro) in culture, it requires only a single exponential in more "mature" cultures (17–21 days in vitro). Our observations on autaptic cultures ($\sim$14 days in vitro) would be consistent with the MK-801 data on more mature cultures.

Our results confirm previous findings (Ryan and Smith, 1995) that, on average, approximately 0.5% of the total recycling pool of synaptic vesicles is released by each action potential. We find that the variability in P_r (as

defined by the initial destaining rates) between synapses yielded a coefficient of variation of 33%. Recent electrophysiological studies have used postsynaptic responses to hypertonic or hyperkalemic solutions to probe the size of the available pool of vesicles at cultured hippocampal synapses (Liu and Tsien, 1995; Stevens and Tsujimoto, 1995). These results suggest a functional pool size on the order of 100 synaptic vesicles. The size of the anatomically defined vesicle pool in cultured hippocampal synapses is unclear but is suggested to be ~100 vesicles (Malgaroli, 1994). If the recycling pool of vesicles labeled by FM1–43 is similar to those defined above, the P_r of release sites would be approximately 0.5. However, a serial reconstruction study of synapses from rat hippocampus has found that some boutons can contain several hundred vesicles (Harris and Sultan, 1995). Thus, the FM1–43 measurements may agree with previous studies (Allen and Stevens, 1994), suggesting that transmitter release is unreliable ($P_r < 1$), but our conclusion depends on knowing the number of synaptic vesicles per bouton.

Although baclofen potently inhibited FM1–43 destaining in single cells and mass cultures during 2–4 Hz stimulation, we observed that destaining during 20 Hz stimulation was much less affected. The inhibition of EPSCs was also frequency dependent. These results are consistent with the increase in paired-pulse facilitation that has been reported for many neuromodulators that inhibit transmitter output (i.e., Manabe et al., 1993). Frequency-dependent presynaptic inhibition has similarly been reported in central and peripheral synapses during high frequency trains of stimulation (Bennett and Lavidis, 1980; Pennartz and Lopes da Silva, 1994; Shen and Horn, 1996).

Several factors may contribute to the apparent ability of high frequency stimulation to overcome presynaptic inhibition. One possibility could be that baclofen is less effective at inhibiting presynaptic calcium channels at high stimulation rates. This seems unlikely since other G-protein-coupled receptors can inhibit calcium currents evoked at high frequencies with voltage pulse protocols simulating action potential waveforms (Toth and Miller, 1995). If baclofen acts by inhibiting a distinct calcium channel subtype, another possibility is that other baclofen-insensitive calcium channels may contribute to transmitter release during high frequency stimulation. Two other classes of explanation seem to us to be more plausible. One invokes the cooperativity of intracellular calcium action on exocytosis and the increase in "residual" calcium in the nerve terminal during high frequency stimulation. As suggested for the modulation of paired-pulse facilitation, an increase in residual $[Ca^{2+}]_i$ would reduce the apparent dependence of transmitter release on action potential-induced calcium influx (Zucker, 1989). The other explanation considers that during rapid stimulation of a terminal, several steps in vesicle recycling may become rate limiting (endocytosis, refilling, and redocking). It may be that during rapid repetitive stimulation, transmitter release falls to the lower rate allowed by these steps and becomes much less sensitive to increases or decreases of calcium entry.

FM1–43 measurements of endocytosis have determined that membrane retrieval occurs on a relatively slow time scale ($t_{1/2}$ ~20–30 s) in hippocampal neurons (our results; Reuter and Porzig, 1995; Ryan and Smith, 1995; Ryan et al., 1996a). We have shown that $GABA_B$ receptor activation does not inhibit membrane retrieval over a 60 s interval following a brief exocytotic stimulus. Indeed, the rapid onset and offset of $GABA_B$ receptor-mediated presynaptic inhibition that we and Pfrieger et al. (1994) have observed is consistent with a rapid membrane-delimited action on a target(s) in the nerve terminal (Hille, 1992). Our results suggest that down-regulation of the available pool of synaptic vesicles via inhibition of the endocytic phase of vesicle recycling is not a mechanism of $GABA_B$ receptor-mediated inhibition.

The precise molecular steps underlying presynaptic inhibition are still under much debate. Considerable evidence points to a modulation of presynaptic calcium channels (Wu and Saggau, 1994; Doze et al., 1995; Dittman and Regehr, 1996). However, because baclofen reduces mEPSC frequency, it has been proposed that inhibition of evoked excitatory synaptic transmission is in part (Dittman and Regehr, 1996) or largely (Scanziani et al., 1992; 1995) independent of presynaptic calcium channel modulation. It is useful to recognize, however, that the relationship of spontaneous transmitter release to nerve-evoked release is unclear. One possibility that could reconcile these results is based on the extensive interactions between N-type calcium channels and proteins implicated in vesicle release, including syntaxin, SNAP-25, and VAMP–synaptobrevin (Calakos and Scheller, 1996; Sheng et al., 1996). The machinery needed for vesicle docking and fusion and the calcium channels mediating release may form a supramolecular complex at the active zone. In this view, G-protein-mediated, membrane-delimited modulation of the calcium channels (Hille, 1994) could lead to conformational changes in the supramolecular fusion machinery. Thus, nerve-evoked and calcium-independent transmitter release could be simultaneously modulated by actions on the presynaptic calcium channel.

Experimental Procedures

Standard methods were used to establish dissociated monolayer cell cultures of regions CA1–CA3 (Bekkers and Stevens, 1990). Briefly, neonatal rats (1–5 days old) were anesthetized (by Methoxyflurane) and decapitated. Hippocampal regions CA1–CA3 were enzymatically dissociated with papain (10–20 U/ml), triturated to the level of single cells, and plated onto cover slips coated with poly-D-lysine (0.1 mg/ml)/collagen (0.5 mg/ml). Dissociated neurons were also plated on microislands formed by small droplets of poly-D-lysine/collagen applied to agarose (0.15%)-coated coverslips (Bekkers and Stevens, 1991). Culture medium contained minimum essential medium supplemented with glucose (20 mM), 5%–10% fetal bovine serum (GIBCO), penicillin/streptomycin (50 U/ml, GIBCO), and MITO+ Serum Extender (1 μl/ml, Collaborative Research). After 3 days in culture, the standard medium was replaced with one containing B27 medium supplement (2%, GIBCO) and cytosine-β-arabinofuranoside (5 μM). Hippocampal cell cultures were used 8–28 days after plating.

For electrical and optical recordings, coverslips were transferred to a recording chamber continually perfused with a Ringer solution containing (in mM): 138 NaCl, 2.5 KCl, 10 HEPES, 2 Mg_2SO_4, 2 $CaCl_2$, and 20 glucose (pH 7.4 with NaOH). Picrotoxin (100 μM), D-amino-5-phosphonovaleric acid (APV, 25 μM), and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μM) were added to block postsynaptic

inhibitory and excitatory transmission. CNQX was omitted when autaptic synaptic responses were recorded. Patch pipettes (2–4 MΩ resistance) contained (in mM): 140 K $MeSO_4$, 4 NaCl, 10 HEPES, 3 Mg ATP, 0.2 NaGTP, and 0.2–5 EGTA (pH 7.4). Series resistance, which was <10 MΩ, was routinely compensated by >80%. Autaptic EPSCs were evoked from a holding potential of −70 mV by a 1–2 ms voltage step to +10 mV to generate an unclamped action current. Synaptic currents were recorded with an Axopatch 1–D amplifier (Axon Instruments) and filtered at 1 kHz. Currents were digitized (4 kHz) and analyzed using BASIC-FASTLAB (Indec Systems). All experiments were performed at room temperature (22–25°C).

Quantitative fluorescence measurements were made using a 12 bit cooled-CCD camera (Princeton Instruments) and the Metamorph software suite (Universal Imaging). Neurons and processes were viewed with a Nikon Diaphot epifluorescence microscope (× 40 oil immersion lens, 1.3 NA) and illuminated with a 75 W xenon lamp through 0.3 or 1.0 neutral density filters. Fluorescence imaging was done with 475–490 nm excitation and 525–550 nm emission filters and exposure times of 0.4 or 1 s/image. Solutions were applied locally via a multibarrel perfusion system controlled by solenoid valves. Imaging of dye-containing solution confirmed that solution exchange over the entire field of view was complete within 1–2 s.

Loading of presynaptic boutons with the fluorescent styryl membrane probe FM1–43 followed previously described methods (Reuter, 1995; Ryan and Smith, 1995). FM1–43 (10–15 μM, Molecular Probes) was added to the superfusing solution, and action potentials were generated by field stimulation (1 ms duration, ~20 V/cm) at 10 or 20 Hz for 20–30 s, applied via platinum electrodes placed on opposite sides of the recording chamber. This stimulation strength was two to three times greater than the threshold for reliably eliciting action potentials from somatic recordings of neurons in the field of view. For experiments in single cells, loading was achieved by the same stimulation used to evoke EPSCs applied at 4 Hz for 60 s. Cells were left in the FM1-43-containing solution for at least 1 min following stimulation to allow for the endocytosis of synaptic vesicles (Ryan et al., 1996a). This was followed by washing of the cells in dye-free Ringer solution for 5 min before destaining measurements.

Functional synapses were identified as bright fluorescent puncta that released FM1–43 during stimulation (2–20 Hz). Regions of interest, typically 1.5 × 1.5 μm^2, were selected to overlap the largest portion of individual fluorescent spots while including as little background area as possible. Images were acquired every 2 or 4 s. The boundaries of the dye-stained region were demarcated for the first image, and fluorescent intensity was averaged over the pixels within the same region for all subsequent images. On average, 10 synapses were selected from each field of view for analysis. Intensity data were corrected for fading (typically 10%/120 s experiment) using an exponential or linear time course derived from nonpunctate, nonreleasable labeling. Dye release declined exponentially during maintained stimulation (Figure 6). Exponential depletion of fluorescence has been interpreted to reflect the entry of nonlabeled recycled vesicles into the releasable pool (Betz and Bewick, 1993). In such a case, the FM1–43 labeled pool (F) decays according to $F_0 \exp(-f \times AP)$ where F_0 is the initial fluorescence before stimulation, f is the destaining rate constant, and AP represents the number of stimuli delivered. The instantaneous destaining rate constant (f) was calculated as $\ln(F_1/F_2)/\Delta AP$ where F_1 and F_2 are values of relative FM1–43 fluorescence, and ΔAP represents the number of stimuli delivered over the time interval separating F_1 and F_2. Rate constants determined in this manner reflect the rate of exocytosis. Data are shown as mean ± SEM. Coefficient of variation = SD/mean.

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